METHOD OF GENETIC MODIFICATION OF A WILD TYPE VIRAL SEQUENCE

Field of the invention

The present invention is related to a method of genetic modification of a wild type viral sequence, for reducing or suppressing deleterious properties of plants or plant cells transformed by said wild type viral sequence.

The present invention is also related to the modified viral sequence obtained by said method, and to the plant and the plant cell comprising said modified viral sequence.

Background of the invention and state of the art

The widespread viral disease of the sugar beet plant (*Beta vulgaris*) called Rhizomania is caused by a furovirus, the beet necrotic yellow vein virus (BNYVV) (1, 2) which is transmitted to the root of the beet by the soilborne fungus *Polymyxa betae* (3).

The disease affects significantly acreages of the area where the sugar beet plant is grown for industrial use in Europe, USA and Japan and is still in extension in several places in Western Europe (4, 5).

Since 1986, number of reports and publications have described the use of isolated viral nucleotidic sequences expressed in plants to confer a high level of tolerance against a specific infectious virus or even to confer a broad spectrum type of resistance against a number of related viruses (6, 7, 8). One of the most documented viral resistance strategy based on genetic engineering, in many cultivated species such as potato, squash, cucumber or tomato, is the use of the viral nucleotidic sequence which under the control of plant regulatory elements, encodes the coat-protein of the target virus (9).

However, in coat-protein mediated resistance, the expression of a certain level of resistance in the transgenic plant might be attributed to different mechanisms such as RNA co-suppression and not necessarily to the production of the protein sequence.

In general, the virus sequence will be transformed in an appropriate cell or tissue culture of the plant species using an *Agrobacterium* mediated transformation system or a direct gene transfer method according to the constraints of the tissue culture or cell culture method which can be successfully applied in a given species. A whole plant will be regenerated and the expression of the transgene will be characterised.

Though sugar beet is known as a recalcitrant species in cell culture, limiting the extent of practical genetic engineering applications in that species, there are number of isolated reports of successful transformation and regeneration of whole plants (38). A few examples of engineering tolerance to the BNYVV by transforming and expressing the BNYVV coat-protein sequence in the sugar

beet genome have also been published (11, WO91/13159) though they rarely report data on whole functional transgenic sugar beet plants (12). In particular, reports show limited data on the level of resistance observed in infected conditions with transgenic sugar beet plants transformed with a gene encoding a BNYVV coat-protein sequence (13, 14).

A complete technology package including a sugar beet transformation method and the use of the expression of the BNYVV coat-protein sequence as resistance source in the transgenic sugar beet plant obtained by said transformation method has been described in the Patent Application WO91/13159.

Based on the information published, it can not be concluded that the coat-protein mediated resistance mechanism provides any potential for conferring to the sugar beet plant a total immunity to the BNYVV-infection by inhibiting completely the virus multiplication and diffusion mechanisms. To identify a resistance mechanism which significantly blocks the spread of the virus at the early stage of the infection process would be a major criteria of success to develop such a transgenic resistance. In addition, such resistance would diversify the mechanisms of resistance available.

Because the disease is shown to expand in many countries or areas, at a speed depending upon the combination of numerous local environmental and agricultural factors, there is a major interest to diversification and improvement of the genetic resistance mechanisms which may, alone or in combination, confer a stable and long lasting resistance strategy in the current

and future varieties of sugar beet plants which are grown for industrial use.

The genome of beet necrotic yellow vein furovirus (BNYVV) consists of five plus-sense RNAs, two of which (RNAs 1 and 2) encode functions essential for infection of all plants while the other three (RNAs 3, 4 and 5) are implicated in vector-mediated infection of sugar beet (*Beta vulgaris*) roots. Cell-to-cell movement of BNYVV is governed by a set of three successive, slightly overlapping viral genes on RNA 2 known as the triple gene block (TGB), which encode, in order, the viral proteins P42, P13 and P15 (gene products are designated by their calculated M_r in kilodalton).

In the following description, the TGB genes and the corresponding proteins will be identified by the following terms : TGB-1, TGB-2, TGB-3 or by their encoded viral protein number P42, P13 and P15. TGB counterparts are present in other furoviruses and in potex-, carla- and hordeiviruses (15, 18, 19, 20, 21 and 22). In the enclosed table 1 are represented viruses having a TGB-3 sequence, the molecular weight of TGB-3 of said viruses, their host and references.

It has been shown previously that independent expression of P15 from a viral-RNA replication species known as a "replicon", derived from BNYVV RNA 3, inhibits infection with BNYVV by interfering cell-to-cell movement (16).

In order to introduce a virus comprising a TGB-3 nucleic acid sequence into a plant cell or a plant, it has been proposed to incorporate a nucleic acid construct comprising said TGB-3 nucleic acid sequence

operably linked to one or more regulatory sequences active in said plant (WO98/07875).

However, while expression of wild type TGB-3 viral sequence in a transgenic plant allows the blocking of said viral infection, the presence of said wild type sequence may induce deleterious effects on the agronomic properties of transformed plants or plant cells.

Aims of the invention

The present invention aims to provide a new method for inducing a genetic modification of a wild type viral sequence involved in the multiplication and diffusion mechanisms of virus infecting plants, in order to reduce or suppress the possible deleterious effects upon plants or plant cells transformed by said viral sequence.

Another aim of the present invention is to provide a method to obtain such a modified viral sequence which blocks virus infection when it is incorporated into a plant or a plant cell.

Summary of the invention

The present invention is related to a method of genetic modification of a TGB-3 wild type viral sequence, preferably the BNYVV P15 viral sequence, for reducing or suppressing the possible deleterious effects on the agronomic properties of the transformed plants or plant cells by said TGB-3 viral sequence.

Preferably, said genetic modification is a point mutation which allows the substitution of at least one amino-acid into another different amino-acid of said TGB-3 wild type sequence, preferably the substitution of at

least one amino-acid into another different amino-acid in the BNYVV P15 sequence.

It seems that the function of the TGB-3 wild type sequence in cell-to-cell movement involves at least in part "bridging" interactions between an element of the host plant (preferably a component of the plasmodesmata), and an element of viral origin (preferably another viral protein involved in cell-to-cell movement). Disruption of either the domain of the TGB-3 wild type sequence (which putatively interacts with the host element) or the domain of the TGB-3 wild type sequence (which putatively interacts with the viral element), allows the inhibition of the cell-to-cell movement.

In addition, it seems that said specific mutations in a TGB-3 wild type sequence allow the production of mutants produced in a transgenic plant, which will still interact with the viral element, but not with the host element. These mutants might compete for binding sites on the viral element of the TGB-3 wild type sequence produced in the initial stage of the viral infection, and abort the infection by inhibiting viral movement to an adjacent cell.

Advantageously, the substitution of at least one amino-acid into another different amino-acid of said sequence is made in regions rich in hydrophilic amino-acids usually present at the surface of the protein in its native configuration.

Preferably, the point mutation(s) allow the substitution of one or two amino-acids into one or two different amino-acids.

In the enclosed Table 1, preferred examples of said viruses having a TGB-3 wild type viral sequence,

the molecular weight of the corresponding TGB-3 peptide, their hosts and a reference, are described. The specific wild type P15 nucleotidic and amino-acid sequences of BNYVV are also already described (17).

The above-described point mutations were realised by conventional methods known by the person skilled in the art.

The above mutants containing the point mutation were tested for their ability to promote cell-to-cell movement of a viral mutant (with a dysfunctional TGB-3 sequence, preferably a BNYVV mutant with a dysfunctional P15 gene) when expressed in trans from a replicon. These mutants were incapable of promoting such movement and were tested for their ability to inhibit infection with a co-inoculated wild type TGB-3 virus, preferably co-inoculated with a wild type BNYVV, when the mutant form of the TGB-3 sequence, preferably the P15 gene, was expressed from a replicon.

The Inventors have discovered unexpectedly that the genetic modification method according to the invention (preferably a point mutation) could be used to obtain a modified TGB-3 viral sequence (preferably a modified BNYVV P15 sequence), which is able to block virus infection without producing deleterious effects when incorporated in the genome of a plant or a plant cell.

It is meant by "being able to block viral infection into a plant or a plant cell", the possibility to obtain a high degree of tolerance by the plant or plant cell transformed by said modified TGB-3 viral sequence to said viral infection, in particular the possibility to ensure rapid and total blocking of the virus multiplication and diffusion mechanisms into the plant, preferably the

blocking of the BNYVV virus multiplication and diffusion mechanisms into a sugar beet plant (beta vulgaris), including fodder beet, Swiss Whard and table beet which may also be subjected to said BNYVV infection.

Said tolerance or resistance could be easily measured by various methods well known by the person skilled in the art.

Preferably, the genetic modifications in the TGB-3 wild type viral sequence are point mutations in the portions of said wild type viral sequence involved in the mechanisms of viral cell-to-cell movements.

The present invention is also related to the modified TGB-3 viral nucleotidic and amino-acid sequences obtained (recovered) by said (modification and selection) method, more preferably the BNYVV P15 modified nucleotidic and amino-acid sequences obtained (recovered) by said method.

Preferably, said BNYVV P15 nucleotidic and amino-acid sequences are selected from the group consisting of the following nucleotidic or corresponding amino-acid sequences :

SEQ ID NO 1 :

ATGGTGCTTGTGGTTGCAGTAGCTTTATCTAATATTGTATTGTACATAGTTGCCGGTTGT 60
M V L V V A V A L S N I V L Y I V A G C

GTTGTTGTCAGTATGTTGTACTCACCGTTTTTCAGCAACGATGTTAAAGCGTCCAGCTAT 120
V V V S M L Y S P F F S N D V K A S S Y

GCGGGAGCAATTTTTAAGGGGAGCGGCTGTATCATGGACAGGAATTCGTTTGCTCAATTT 180
A G A I F K G S G C I M D R N S F A Q F

GGGAGTTGCGATATTCCAAAGCATGTAGCCGAGTCCATCACTAAGGTTGCCACCAAAGAG 240
G S C D I P K H V A E S I T K V A T K E

CACGATGTTGACATAATGGTAAAAAGGGGTGAAGTGACCGTTCGTGTTGTGACTCTCACC 300
H D V D I M V K R G E V T V R V V T L T

GAAACTATTTTTATAATATTATCTAGATTGTTTGGTTTGGCGGTGTTTTTGTTCATGATA 360
E T I F I I L S R L F G L A V F L F M I

TGTTTAATGTCTATAGTTTGGTTTTGGTATCATAGATAA 399
C L M S I V W F W Y H R *

SEQ ID NO 2 :

ATGGTGCTTGTGGTTAAAGTAGATTTATCTAATATTGTATTGTACATAGTTGCCGGTTGT 60
M V L V V K V D L S N I V L Y I V A G C

GTTGTTGTCAGTATGTTGTACTCACCGTTTTTCAGCAACGATGTTAAAGCGTCCAGCTAT 120
V V V S M L Y S P F F S N D V K A S S Y

GCGGGAGCAATTTTTAAGGGGAGCGGCTGTATCATGGCCGCGAATTCGTTTGCTCAATTT 180
A G A I F K G S G C I M A A N S F A Q F

GGGAGTTGCGATATTCCAAAGCATGTAGCCGAGTCCATCACTAAGGTTGCCACCAAAGAG 240
G S C D I P K H V A E S I T K V A T K E

CACGATGTTGACATAATGGTAAAAAGGGGTGAAGTGACCGTTCGTGTTGTGACTCTCACC 300
H D V D I M V K R G E V T V R V V T L T

GAAACTATTTTTATAATATTATCTAGATTGTTTGGTTTGGCGGTGTTTTTGTTCATGATA 360
E T I F I I L S R L F G L A V F L F M I

TGTTTAATGTCTATAGTTTGGTTTTGGTATCATAGATAA 399
C L M S I V W F W Y H R *

SEQ ID NO 3 :

ATGGTGCTTGTGGTTAAAGTAGATTTATCTAATATTGTATTGTACATAGTTGCCGGTTGT 60
M V L V V K V D L S N I V L Y I V A G C

GTTGTTGTCAGTATGTTGTACTCACCGTTTTTCAGCAACGATGTTAAAGCGTCCAGCTAT 120
V V V S M L Y S P F F S N D V K A S S Y

GCGGGAGCAATTTTTAAGGGGAGCGGCTGTATCATGGACAGGAATTCGTTTGCTCAATTT 180
A G A I F K G S G C I M D R N S F A Q F

GGGAGTTGCGATATTCCAAAGCATGTAGCCGAGTCCATCACTAAGGTTGCCACCAAAGAG 240
G S C D I P K H V A E S I T K V A T K E

CACGATGTTGACATAATGGTAAAAAGGGGTGAAGTGACCGTTCGTGTTGTGACTCTCACC 300
H D V D I M V K R G E V T V R V V T L T

GAAACTATTTTTATAATATTATCTAGATTGTTTGGTTTGGATGATTTTTTGTTCATGATA 360
E T I F I I L S R L F G L D D F L F M I

TGTTTAATGTCTATAGTTTGGTTTTGGTATCATAGATAA 399
C L M S I V W F W Y H R *

In the following description, the various modified BNYVV TGB-3 sequences will be hereafter called "P15 mutants", identified by the following reference : BNP15-Ala1, corresponding to SEQ ID NO 1, BNP15-Ala4 corresponding to SEQ ID NO 2, BNP15-Asp9, corresponding to SEQ ID NO 3.

The nucleotidic and corresponding amino-acid sequences of SEQ ID NO 1, SEQ ID NO 2 and SEQ ID NO 3 can be compared to SEQ ID NO 4, which is the sequence of the wild type P15 nucleotidic and amino-acid sequence already described (17).

The present invention is also related to the vector comprising said modified nucleotidic sequence possibly being operably linked to one or more regulatory sequence(s) active into a plant or a plant cell. Preferably, said vector is a plasmid comprising already said regulatory sequence(s) active into a plant or a plant cell.

The present invention is also related to a method for inducing a resistance to a virus comprising a TGB-3 sequence, preferably one of the viruses described in the enclosed Table 1, and more preferably the BNYVV virus, said method comprising the following steps :

- preparing a nucleic acid construct comprising a nucleic acid sequence being genetically modified according to the method of the invention and being operably linked to one or more regulatory sequences active into a plant or a plant cell,
- transforming the plant cell with the nucleic acid construct, and
- possibly regenerating the transgenic plant from the transformed plant cell.

Preferably, said method is used for inducing a resistance to the BNYVV into a sugar beet plant or a sugar beet cell. Said method comprises the following steps :

- preparing a nucleic acid construct comprising a modified nucleic acid sequence obtained by the method according to the invention, preferably preparing a nucleic acid construct comprising a nucleic acid sequence selected from the group consisting of SEQ ID NO 1, SEQ ID NO 2 or

SEQ ID NO 3, being operably linked to one or more regulatory sequences active into a plant,

- transforming the sugar beet plant cell with the nucleic acid construct, and
- possibly regenerating the transgenic sugar beet plant from the transformed sugar beet plant cell.

The present invention is also related to the obtained (recovered) transgenic plant or the transgenic plant cell resistant to an infection by a virus comprising a TGB-3 sequence, preferably one of the viruses described in the enclosed Table 1, more preferably the BNYVV virus, said plant or plant cell comprising a nucleic acid construct having a TGB-3 modified nucleic acid sequence, being operably linked to one or more regulatory sequences capable of being active into a plant or a plant cell.

Preferably, said modified nucleic acid sequence is selected from the group consisting of SEQ ID NO 1, SEQ ID NO 2 and SEQ ID NO 3, being operably linked to one or more regulatory sequences active into a plant or a plant cell.

Preferably, the cell is a stomatal cell and the regulatory sequence comprises a promoter sequence and a terminator sequence capable of being active into a plant. Said promoter sequence can be constitutive or could be obtained from a foreigner promoter sequence, and is preferably selected from the group consisting of the 35S Cauliflower Mosaic Virus promoter, and/or the polyubiquitin Arabidopsis thaliana promoter.

Advantageously, the promoter sequence is a promoter which is mainly capable of being active in the root tissue of plants such as the par promoter or the haemoglobin gene from Perosponia andersonii.

A last aspect of the present invention is related to a transgenic plant tissue such as fruit, stem, root, tuber, seed of the transgenic plant according to the invention or a reproducible structure (preferably selected from the group consisting of calluses, buds or embryos) obtained from the transgenic plant or the plant cell according to the invention.

The techniques of plant transformation, tissue culture and regeneration used in the method according to the invention are the ones well known by the person skilled in the art. Such techniques are preferably the ones described in the International Patent Applications WO95/101778, WO91/13159 (corresponding to the European Patent Application EP-B-0517833), WO98/07875, which are incorporated herein by reference.

These techniques are preferably used for the preparation of transgenic sugar beet plants and plant cells according to the invention.

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Table 1

Virus	Size of TGB-3	Host	Reference
Apple stem pitting virus	8 kDa	apple	Jelkman, *J. Gen. Virol.* 75, 1535-1542 (1994)
Blueberry scorch virus	7 kDa	blueberry	Cavileer et al., *J. Gen. Virol.* 75, 711-720 (1994)
Potato virus M	7 kDa	potato	Zavriev et al., *J. Gen. Virol.* 72, 9-14 (1991)
White clover mosaic virus	8 kDa	clover	Forster et al., *Nucl. Acids Res.* 16, 291-303 (1988)
Cymbidium mosaic virus	10 kDa	orchid	Neo et al., *Plant Mol. Biol.* 18, 1027-1029 (1992)
Potato virus X	8 kDa	potato	Rupasov et al., *J. Gen. Virol.* 70, 1861-1869 (1994)
Barley stripe mosaic virus	17 kDa	barley	Gustafson et al., *Nucl. Acids Res.* 14, 3895-3909 (1986)
Potato mop top virus	21 kDa	potato	Scott et al., *J. Gen. Virol.* 75, 3561-3568 (1994)
Peanut clump virus	17 kDa	peanut	Herzog et al., *J. Gen. Virol.* 75, 3147-3155 (1994)
Beet soil-borne virus	22 kDa	Sugar beet	Koenig et al., *Virology* 216, 202-207 (1996)